

Final Report

Effects of Gravity on Insect Circadian Rhythmicity

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CASI

BACKGROUND

Circadian rhythms – endogenous daily rhythmic fluctuations in virtually all characteristics of life – are generated and coordinated by the circadian timing system (CTS; 1-4). The CTS is synchronized to the external 24-hour day by time cues such as the light/dark cycle. In an environment without time cues, the length of an animal's day is determined by the period of its internal pacemaker (τ) and the animal is said to be free-running. All life on earth evolved under the solar day; the CTS exists as an adaptation that allows organisms to anticipate and to prepare for rhythmic environmental fluctuations. All life on earth also evolved under the force of earth's gravitational environment. While it is therefore not surprising that changes in the lighting environment affect the CTS, it is surprising that changes in the gravitational environment would do so. However, recent data from one of our laboratories using the brn-3.1 knockout mouse revealed that this model, which lacks the sensory receptor hair cells within the neurovestibular system, does not respond to exposure to a hyperdynamic environment in the same fashion as normal mice (5). The brn-3.1 mice did not show the expected suppression of circadian rhythmicity shown by control mice exposed to 2G.

Exposure to altered ambient force environments affects the amplitude, mean and timing of circadian rhythms in species from unicellular organisms to man (6). In addition, there is a circadian influence on the homeostatic response to acute 2G acceleration (7, 8) and pulses of 2G can act as a time cue, synchronizing the CTS (9, 10). This is of significance because maintenance of internal and external temporal coordination is critical for normal physiological and psychological function (11-13).

Typically, during adaptation to an increased gravitational environment (+G), an initial acute reaction is followed by adaptation and, eventually, a new steady state (14-16), which can take weeks to months to establish. Until the development of space stations, exposure to microgravity was, of necessity, relatively short in duration. In early spaceflight experiments an organism's internal rhythms often expressed periods that were different from each other, even in the presence of a 24.0 hour light-dark cycle, suggesting that the organism was experiencing internal desynchronization (17, 18). In μ G, the body temperature rhythm was delayed with respect to other body rhythms and to the light-dark cycle in rhesus macaques (19) and man (20, 21). In the absence of a

light-dark cycle, the circadian rhythm of spore formation persisted in *Neurospora crassa*, however, both the variability and average period of the rhythm increased (22). The beetle *Trigonoscelis gigas*, exhibited changes in period during and following 11-13 days in μG (23, 24). Resynchronization of the urinary calcium rhythm following a 180° phase shift of the LD cycle was retarded in rats exposed to μG compared to 1G controls (25).

With the development of the Russian Mir Space Station, long-term controlled microgravity exposure became possible. We recorded activity rhythms from black-bodied Tenebrionid beetles, *Trigonoscelis gigas*, in μG (spaceflight). Each insect was housed individually within an activity monitor (26) and data (activity counts) were collected and stored in five-minute bins. Thirty-two individual activity monitors were housed within each of 2 experimental kits. The beetles within each kit were divided into two groups and the lighting was controlled separately for each group.

METHODS

Hardware (Beetle Kit). The hardware constructed for this experiment (Lockheed-Martin, L & M Electronics) was designed to: collect activity data from a large number of individual insects, store these data, and provide and control the lighting environment. The hardware was a modification of the Russian design flown on COSMOS and has been described previously (28). Briefly, each insect was housed individually within an activity monitor designed along the principle of a revolving door; as the insect walked it pushed the door ahead of it. The "revolving door" was linked to a slotted wheel located beneath the floor of the insect's chamber. An optical emitter/detector system registered movement of the wheel. Data (activity counts) were collected in five-minute bins and stored on solid-state data loggers (Mini-logger, Minimitter, Inc.). The height of the ceiling of each monitor could be adjusted to ensure that the insect would maintain contact with the floor in the microgravity spaceflight environment. Lighting was provided by four LEDs located in the ceiling (Super Bright Green; Hiyoshi Electric). The LED used has a spectral peak at 574 nm with a spectral half-width of 11 nm and provided an average illumination level of 17 lux. The individual activity monitors

were housed within an outer enclosure that also contained the data loggers, batteries, electronics to control the lighting environment and a ventilation system used to provide airflow to each individual beetle during the experiment. An enclosure contained 32 individual activity monitors divided into two groups of 16. The lighting was controlled separately for each group. The Beetle Kit is diagrammed in Figure 1 below.

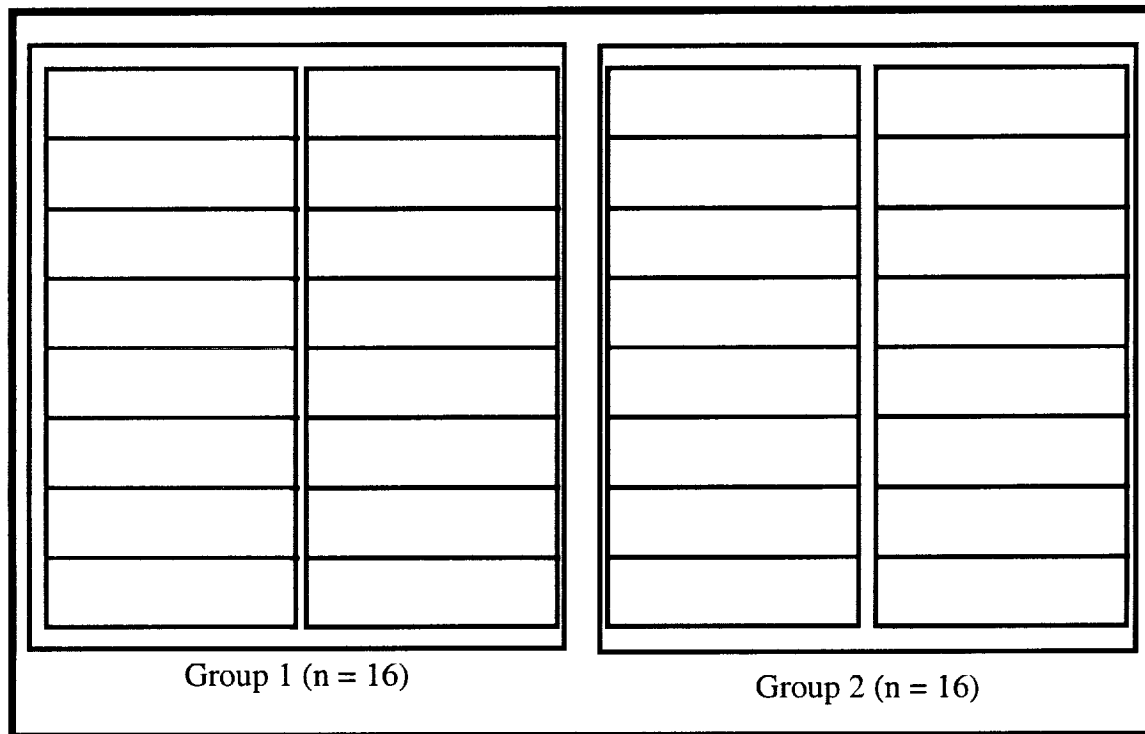


Figure 1. Diagram of the Beetle Kit hardware. The Kit contains four stacks of 8 activity monitors, each of which houses an individual beetle. Ambient lighting is controlled separately for two groups of 16 monitors.

Subjects. Adult beetles (*Trigonoscelis gigas*) were wild-caught in the desert of Turkmenistan in March/April; two hundred insects were collected each year. Where possible, the sex of each insect was determined through behavioral observation. Prior to the experiments, animals were individually housed on sand in plastic containers and provided dry oatmeal *ad lib*.

Selection. In order to assess the quality of each animal's activity rhythm, data were collected for 5 days in constant darkness. The activity rhythms were examined for stability and amplitude. Only animals with rhythms deemed acceptable by three investigators were used in the study. All attempts were made to use an equal number of male and female subjects in each group of the study.

Protocol. Two separate experiments were performed on the Mir Space Station to study the effects of G-level on the CTS. The first experiment examined the tonic effects of light and the second the phasic effects of light. The two lighting protocols are diagrammed in Figure 2.

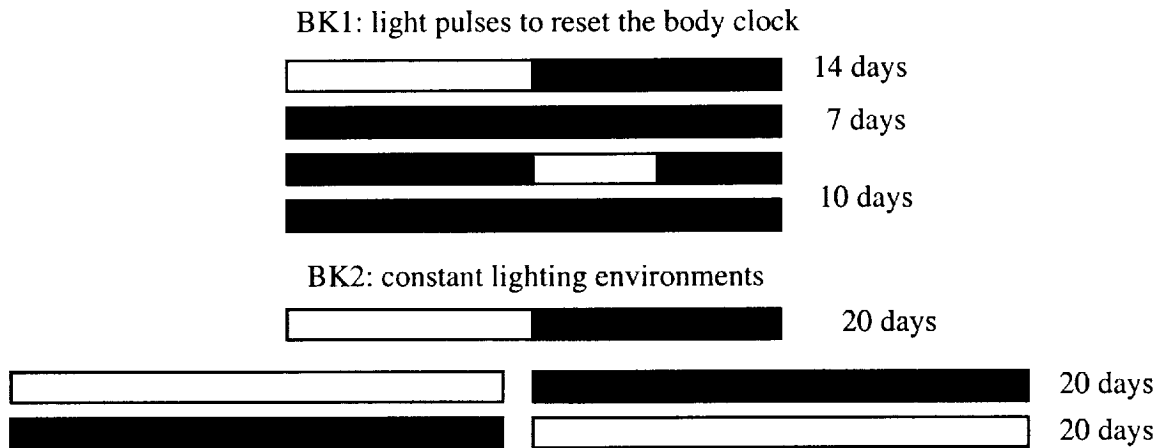


Figure 2. Diagrams of the constant (BK1, top) and phase shift (BK2, bottom) lighting protocols.

Constant light (tonic). This constant lighting protocol would test the effect of ambient light intensity on τ . The lighting protocol for this experiment was a cross-over design of constant light (LL) and constant darkness (DD). The constant lighting schedule began with LD 12:12 (12 hours of light followed by 12 hours of darkness) for 20 days. Group 1 was then exposed to constant light (LL) for 20 days while Group 2 was placed in constant darkness (DD). The two groups were then crossed-over to the opposite lighting condition. This 60-day protocol was repeated with Group 1 receiving DD first and Group 2 LL.

Phase shift (phasic). The second was designed to test the phasic effects of light, a series of light pulses (LPs) was given against a background of darkness. The response of the CTS to a LP depends on the time in the animal's day that

the LP is given. Typically, the pacemaker will reset to a later time (phase delay) in response to a LP given in the first half of the animal's night while a LP given in the second half of the animal's night would evoke the opposite response, a resetting to an earlier time (phase advance). A LP given during the animal's day usually does not reset the pacemaker. The 27-day repeating light pulse protocol consisted of LD 12:12 for 10 days followed by 17 days of DD. On the 7th day of DD, a 6-hour light pulse (LP) was administered. Each time the protocol repeated, the LP was administered at a different time in the animal's day. For the first run, Group 1 received the LP during the first half of their "night" and Group 2 received the LP in the second half of their night. For the second run the groups were crossed over to the opposite night-time LP. During the third repeat both groups receiving the LP during their "day." These three steps were then repeated.

Mir study (μ G). The position of male and females were alternated within the hardware. Beetles were placed into the activity monitors and data recorded for 36 hours. These data were examined and individual insects replaced as necessary. The experiment was placed on the shuttle 20 hours prior to launch.

Three days later, the Space Shuttle docked with the Mir space station and the experiment was transferred to Mir, stowed into a locker, plugged in to Mir power and ventilated. Thereafter, the hardware was ventilated once per week using a manual bicycle pump. After 143 days, the experiment hardware was unplugged from Mir power and returned to earth on the space shuttle on October 6, 1997. The experiments were to have been conducted over this four-month period. Unfortunately, 45 days after the launch of the experiment, there was an accidental collision between Mir and an unmanned supply vessel which resulted in a partial decompression and a loss of power to the Station. Following the accident, on several occasions, ambient temperature fell to between 9° and 20 °C for periods of time from 10-14 days, disrupting the insects' circadian rhythmicity.

2G study. The following May a study was performed at 2G and 1G using the constant lighting protocol and the data were compared with those recorded on Mir in μ G. A 2G gravito-inertial field was produced through the use of a 4.5 meter diameter centrifuge at the Chronic Acceleration Research Unit at the University of California Davis. The centrifuge is configured such that the

increased force vector is produced perpendicular to the floor of the animal enclosure.

Data and Analysis. Results were compared between groups using ANOVA and a post-hoc Tukey Kramer test.

Constant light. Each experiment began with 32 beetles as subjects. However, some insects became arrhythmic or immobile during the experiment. In μ G, the subjects consisted of 15 females and 17 males. Of these, useable data were gathered from 12 females and 15 males. Under 1G, data were provided by 12 of 16 females and 12 of 16 males. During the 2G experiment, one of the data loggers failed, reducing our n to 28, of these useable data were collected from 7 of 16 females and 9 of 12 males. The period of the free-running activity rhythm was determined using the periodogram technique (27). Data from the last ten days of either LL or DD were used; the clock should have established a steady-state free run by this point.

Phase shift. The concept of Circadian Time (CT) is used to compare rhythms between individuals with different τ 's. A free-running animal's day is divided into 24 equal Circadian hours. CT 0 is designated as dawn (lights-on in LD 12:12)

and CT 12 is dusk (lights-off in LD 12:12). Phase shifts were determined as follows: an education (average daily waveform) was constructed using the data from the last three days of DD prior to the light pulse. The time of the onset of activity was taken as the first consistent point above the mean level. The period of the rhythm was determined and used to project the anticipated time of activity onset at the end of DD. An education was made of these data and the difference between the predicted and the actual onsets of activity was taken as the phase shift.

RESULTS

Representative data collected on board the Mir space station under each lighting protocol are shown in Figure 3. The data are presented as double-plotted actograms. In this format successive days of data are plotted both below and beside one another. This form of presentation allows the viewer to track the movement of the activity rhythm over time. Times of low ambient temperature are indicated. During these times, the animals often ceased activity or became arrhythmic.

Figure 3. Activity data from two representative subjects collected on the Mir Space Station. Activity is plotted as a downward line vs. time of day. Data are presented in a double-plotted actogram format; successive days of data are plotted both beside and below one another. This allows the viewer to track the movement of the activity rhythms over time. The animal in the left panel was under the phase shift protocol and the animal in the right panel was under constant lighting protocol (see Methods for details). The time of launch is indicated. Times of non-nominal ambient temperature conditions are indicated by the line between the two panels.

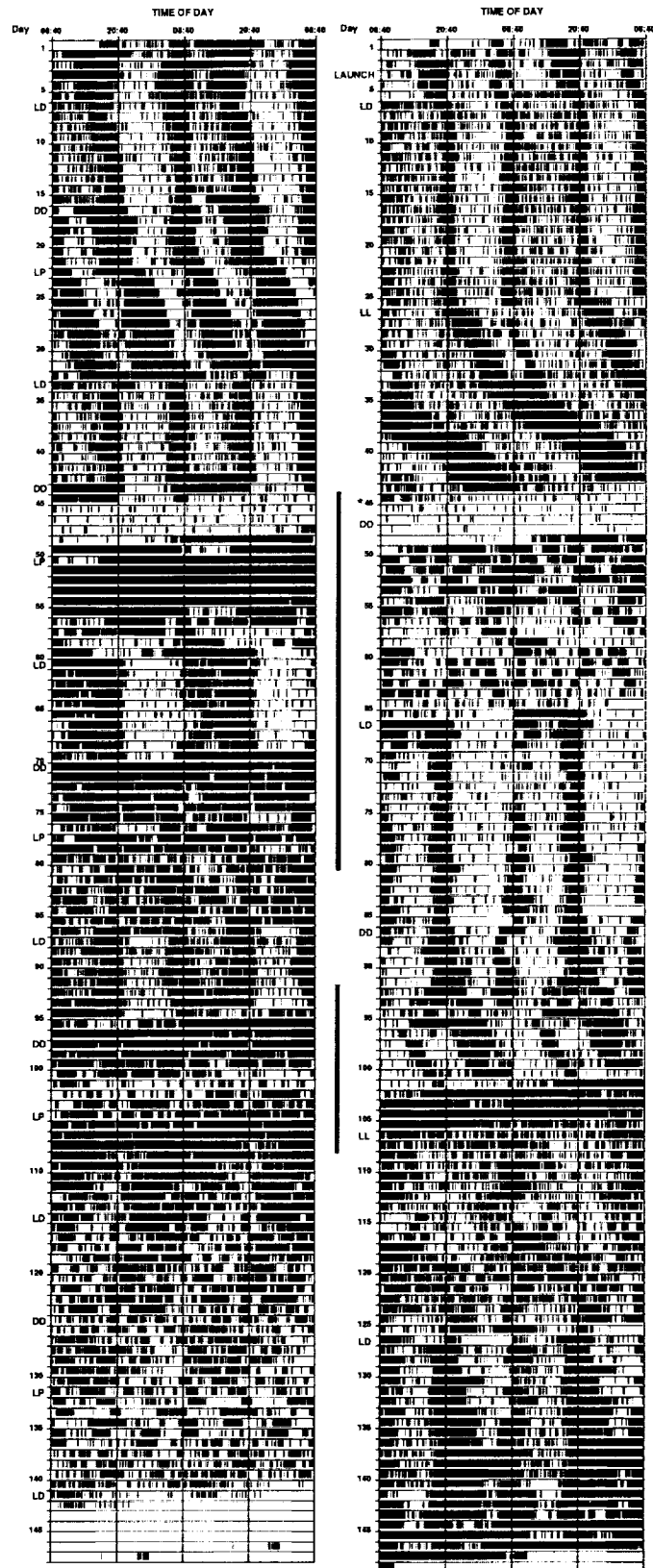


Figure 4 (below) gives an expanded view of the initial data from Figure 3.

The animal in the left panel was in the light pulse protocol; its activity rhythm phase delayed in response to the light pulse given on day 23 of the record. The animal in the right panel was under the constant lighting protocol; the free-running period of its rhythm in constant light (LL) was 26.75 hours.

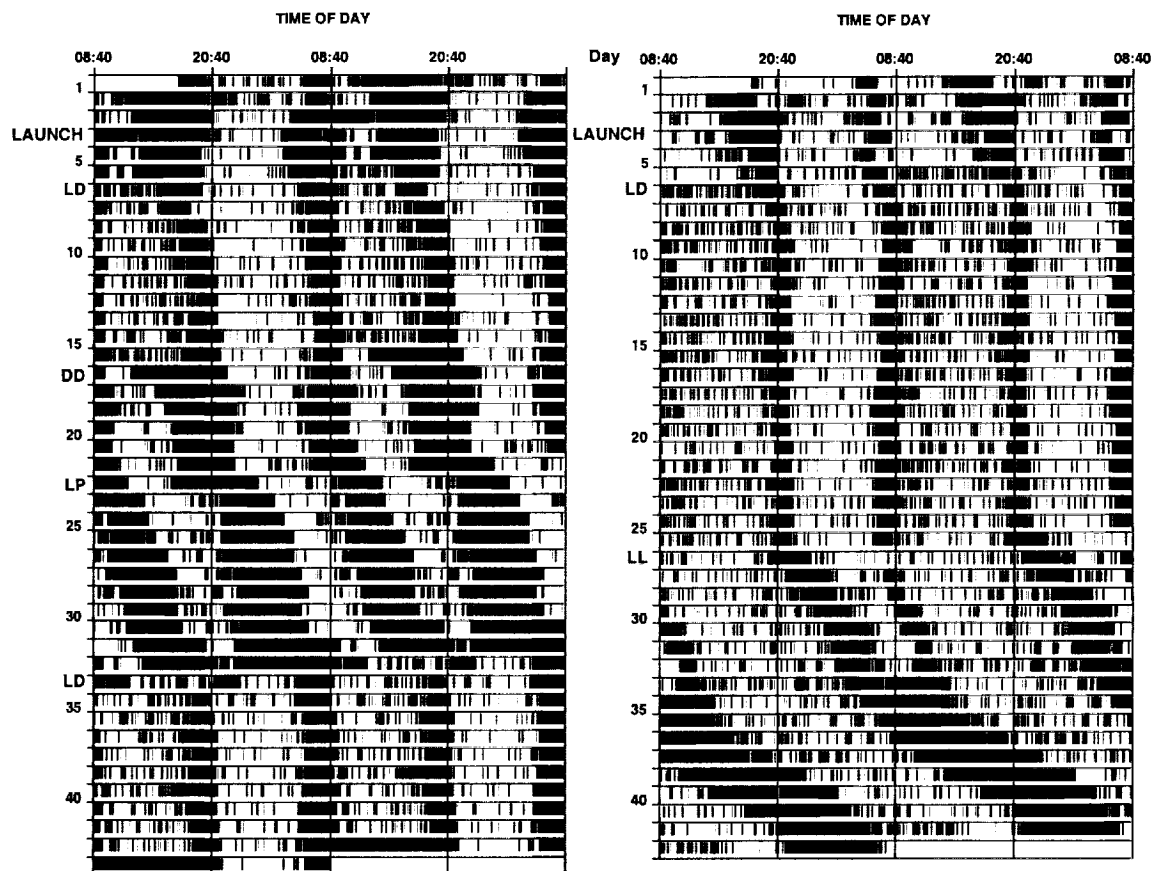


Figure 4. Data from days 1-45 of the study (prior to the collision) are plotted in actogram format. These data are partial records taken from the complete data sets plotted in Figure 3.

Constant light. Data from a representative subject in each constant lighting condition at each G-level are shown in Figure 5. Data collected under LL are in the top panels and under DD in the lower panels. Data collected on Mir (μ G) are in the left panels, under 1G in the center, and at 2G produced via centrifugation on the right. Although a bimodal activity pattern is typical for this species in a light-dark cycle, a change to a single peak pattern is often seen in constant conditions (28).

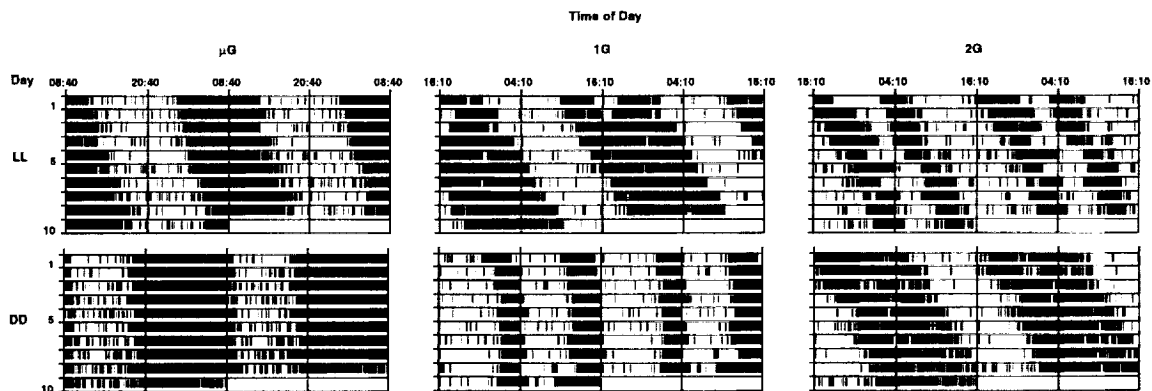
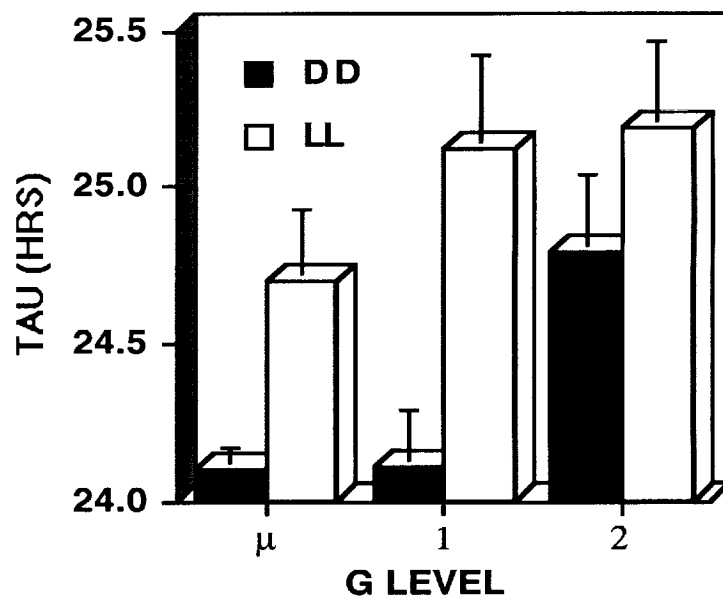


Figure 5. Double-plotted actograms of activity data collected under constant light (LL, upper panels) and constant darkness (DD, lower panels) at each G-level (left = μ G, center = 1G and right = 2G).

Periods were compared among G-levels and lighting conditions. ANOVA was used to determine differences between the periods expressed under different conditions. There was an effect of G-level on τ ($F = 6.473$; $p < 0.03$). A post-hoc Tukey-Kramer test was used to compare data between G-levels; the average τ in

microgravity (24.33 ± 0.70 hours) was significantly shorter from that recorded at 2G (25.27 ± 1.07 hours) but did not differ from that expressed at 1G (24.61 ± 0.90 hours). There was no significant difference between the periods expressed in 1G and 2G. The lighting environment also had a significant effect on period ($F = 5.603$, $P < 0.02$); τ_{LL} (24.91 ± 0.93 h) was longer than τ_{DD} (24.47 ± 0.94 h). At each G-level the average τ was longer in LL than in DD (34). Average τ for each gravitational level and lighting condition are plotted in Figure 6. The difference between τ_{LL} and τ_{DD} was greater at 1G (1.01 hours) than at either μ G (0.60 hours) or 2G (0.40 hours). There was no gender difference in τ (35) and no interaction between any of the variables.

Figure 6. Histogram of average tau (\pm s.e.m.) for each lighting condition and G-level.



Phase shifts. While we do not have comparable data collected at 1G, we were able to demonstrate phase shifts in response to light pulses in the μG environment. These are plotted vs. time of the light pulse in Figure 7.

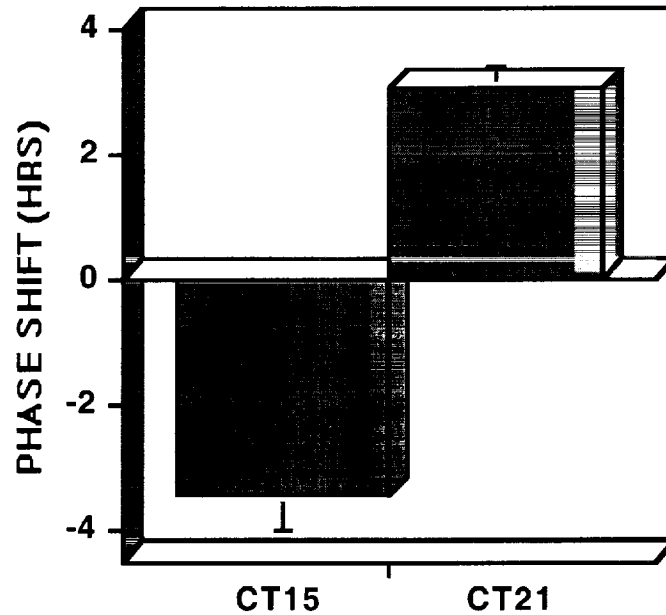


Figure 7. Histogram of the average phase shift (\pm s.e.m.) generated in response to a 6-hour light pulse presented in the first half of the night (CT 15) or the second half of the night (CT 21).

In μG , beetles responded to a LP given during the first half of the night (centered on CT 15) with an average phase delay of 3.45 ± 0.54 hours and to a LP presented during the second half of the night (centered on CT 21) with an average phase advance of 3.05 ± 0.33 hours. The direction of the phase shifts and the magnitude of the phase advances were similar to what was seen in

experiments conducted on earth, however, larger phase delays were seen on Mir than could be elicited using comparable light pulses at 1G (29, 30).

DISCUSSION

Data from the COSMOS rhesus bioflights (19) and data recorded from a human (20, 21) both show a phase delay in the body temperature rhythm during spaceflight, which could indicate a lengthening of the clock's period. These studies were conducted in a light-dark cycle. The period of the rhythm of spore formation in *Neurospora* did not change significantly in spaceflight, although there was a tendency for the periods to be longer (22). Previous studies on *T. gigas* resulted in mixed responses to short-term μG exposure; μG exposure caused an increase, decrease or no change in τ (23, 24). However, these studies were all of too short a duration for the subjects to establish steady-state free-running rhythms.

In all G-levels, the free-running period of the activity rhythm was longer in LL than in DD. This response, an increase in τ with an increase in light intensity, has been seen in many species. Animals that do not follow this pattern, such as reptiles and birds, are those that have photoreceptive pineal glands.

These data are the first recorded from animals who had established a free-running rhythm in μG . In comparison with the data collected at 1 and 2G, they

reveal that the gravitational environment can affect the period of the circadian clock. Given that the CTS is vital to normal physiology, behavior and performance, this fact has implications for long duration spaceflights.

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